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Molecular phylogeny of the Western Palaearctic *Cordulegaster* taxa (Odonata: Anisoptera: Cordulegastridae)

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Although Odonata are a key component of many freshwater ecosystems, their taxonomy and evolutionary history is still far from being well resolved. In the present study, we report the first molecular phylogeny for the Western Palaearctic *Cordulegaster* genus (Odonata: Anisoptera: Cordulegastridae). We sequenced fragments of both mitochondrial and nuclear genes [cytochrome *c* oxidase I (COI) and Internal Transcribed Spacer-1 (ITS-1)] from eight species and 13 subspecies, from western, southern and central Europe, Turkey, and Morocco. Our data support the existence of two major groups corresponding to the traditional *boltonii*- and *bidentata*-groups. Both groups are monophyletic based on COI sequences and the distinctiveness of *Cordulegaster princeps*, *Cordulegaster trinacriae*, *Cordulegaster picta* and *Cordulegaster heros* relative to *Cordulegaster boltonii*, and *Cordulegaster helladica* and *Cordulegaster insignis* relative to *Cordulegaster bidentata*, is confirmed. All species are also monophyletic for ITS-1, with the exception of *Cordulegaster helladica buchholzi*, which shares the haplotype with *C. insignis*. Although moderate levels of genetic diversity were found within *C. boltonii*, there was no clear separation among the four subspecies, with the exception of the populations of *Cordulegaster boltonii algirica* from North Africa. Similarly, no genetic differentiation was found between the two subspecies of *C. bidentata*, *Cordulegaster bidentata bidentata* and *Cordulegaster bidenta sicilica*. *Biological Journal of the Linnean Society*, 2014, **111**, 49–57.

ADDITIONAL KEYWORDS: *Cordulegaster bidentata* – *Cordulegaster boltonii* – Europe – mitochondrial DNA – Morocco – nuclear DNA – Turkey.

INTRODUCTION

Odonata are a key component of many freshwater ecosystems. Their dependence on freshwater for reproduction, their role as predators, and their sensitivity to changes in the environment make them valuable indicators of the overall wetland habitats quality. Studies on Odonata have increased significantly in the last decades and they are currently regarded as model organisms for ecological and evolutionary research (Córdoba-Aguilar, 2008). Nevertheless, the Odonata taxonomy and evolutionary history is still far from being well resolved, even in

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Europe where the number of species is small and where a large number of studies have been conducted (Dijkstra & Kalkman, 2012).

The genus *Cordulegaster* (Odonata: Anisoptera: Cordulegastridae) is a good example of a phylogenetically poorly understood group. With a Holarctic distribution, this genus comprises approximately 30 species. Regardless of the controversial placement and/or validity of several species, to date, only the validity of *Cordulegaster bilineata* has been tested with molecular markers (Pilgrim, Roush & Krane, 2002). *Cordulegaster* has eight species currently recognized in the Western Palaearctic, which are traditionally divided into two groups: the *boltonii*- and the *bidentata*-groups (Boudot, 2001). These groups differ in morphology and ecological traits. Morphological distinctness is found in the superior abdominal appendages of males and in the ovipositor of females. Ecologically, *boltonii*-group habitats vary from hill and mountain springs and from brooks to rivers, whereas the species of the *bidentata*-group are confined to upper courses of brooks as well as to seepage and spring habitats. Despite their widespread distribution and complex subspecific division, the validity of some species and some subspecies is still questioned and genetic diversity within Western Palaearctic species of *Cordulegaster* remains unknown (Boudot *et al.*, 2009). This obscures conservation priorities and hampers the development of adequate conservation measures because, of the eight species in the Western Palaearctic, three are classified with threatened categories and four are Near Threatened (Kalkman *et al.*, 2010; Samraoui *et al.*, 2010). In the present study, we sequenced the internal transcribed spacer 1 (ITS-1) and part of the mitochondrial (mt)DNA cytochrome *c* oxidase I (COI) gene in order to determine: (1) the phylogenetic relationships between all the *Cordulegaster* species of the Western Palaearctic region and (2) the genetic distinctiveness of most subspecies.

MATERIAL AND METHODS

Specimens examined

A total of 112 samples of all Western Palaearctic *Cordulegaster* species covering the majority of species with ranges from Morocco and southern, western and central Europe and south-western Anatolia were analyzed (Fig. 1, Table 1, see also Supporting information, Table S1). Representatives of 13 subspecies, from 59 different locations, were assigned morphologically to subspecific taxa *sensu* Boudot (2001) and Boudot & Jacquemin (1995), whenever possible (Table 1 and Fig. 1). As outgroups, representatives of *Anotogaster sieboldi* Selys, 1854, *Chlorogomphus okinawensis* Ishida, 1964, *Chlorogomphus brunneus* Oguma, 1926, *Chlorogomphus iriomotensis* Ishida, 1972, *Onychogomphus forcipatus* (Linnaeus, 1758) and *Anax imperator* Leach, 1815 were selected (Table 1).

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from a leg of each individual in accordance with a high-salt protocol (Sambrook, Fritsch & Maniatis, 1989). Two partial DNA fragments, mtDNA COI and ITS-1 were amplified using the polymerase chain reaction (PCR). The COI was amplified using the LCO1490 and HC02198 primers (Folmer *et al.*, 1994) producing approximately 700-bp fragments and the ITS-1 with 18S-forward and 5.8S-reverse primers (Pilgrim *et al.*, 2002), producing approximately 900-bp fragments. The PCR conditions (25-μL reactions) were: 19 μL of H_2O, 2.5 μL of 10× Promega Buffer B, 0.5 μL of 10 mM of each primer, 1.5 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM dNTPs, 0.1 μL of Promega *Taq* DNA polymerase, and 0.5 μL of 100 ng per μL DNA template. The cycle parameters were: initial denaturation at 94 °C for 3 min, denaturation at 94 °C (30 s), annealing at 56 °C (45 s) for both COI and ITS-1, and extension at 72 °C (45 s) repeated for 35 cycles, with a final extension of 5 min at 72 °C. Amplified DNA fragments were purified and sequenced (most with both primers) using the same primers as in the PCR, following ABI PRISM BigDye Terminator protocols. Sequences were visualized on a 310 Applied Biosystem DNA Sequencing Apparatus. All sequences were submitted to GenBank (Table 1).

Phylogenetic analysis

COI alignment was performed manually using BIOEDIT, version 5.0.9. (Hall, 1999). Only the different COI haplotypes were included in the phylogenetic analyses. The alignment of the *Cordulegaster* haplotypes was analyzed using maximum likelihood (ML) and Bayesian inference (BI) methods, including the outgroups. The best-fit model of nucleotide substitution evolution under corrected Akaike information criterion was estimated using JMODELTEST, version 0.1.1 (Posada, 2008). Model GTR + G was chosen and used in the ML phylogenetic analyses. ML trees were built in PHYML (Guindon *et al.*, 2010) with 1000 bootstrap replicates and searching for the best-scoring ML tree. Phylogenetic BI was performed using MrBayes, version 3.1.2 (Ronquist & Huelsenbeck, 2003). Two independent runs of 10^7 generations were sampled at intervals of 100 generations, producing a total of 100 000 trees. Burn-in was determined upon convergence of log likelihood and

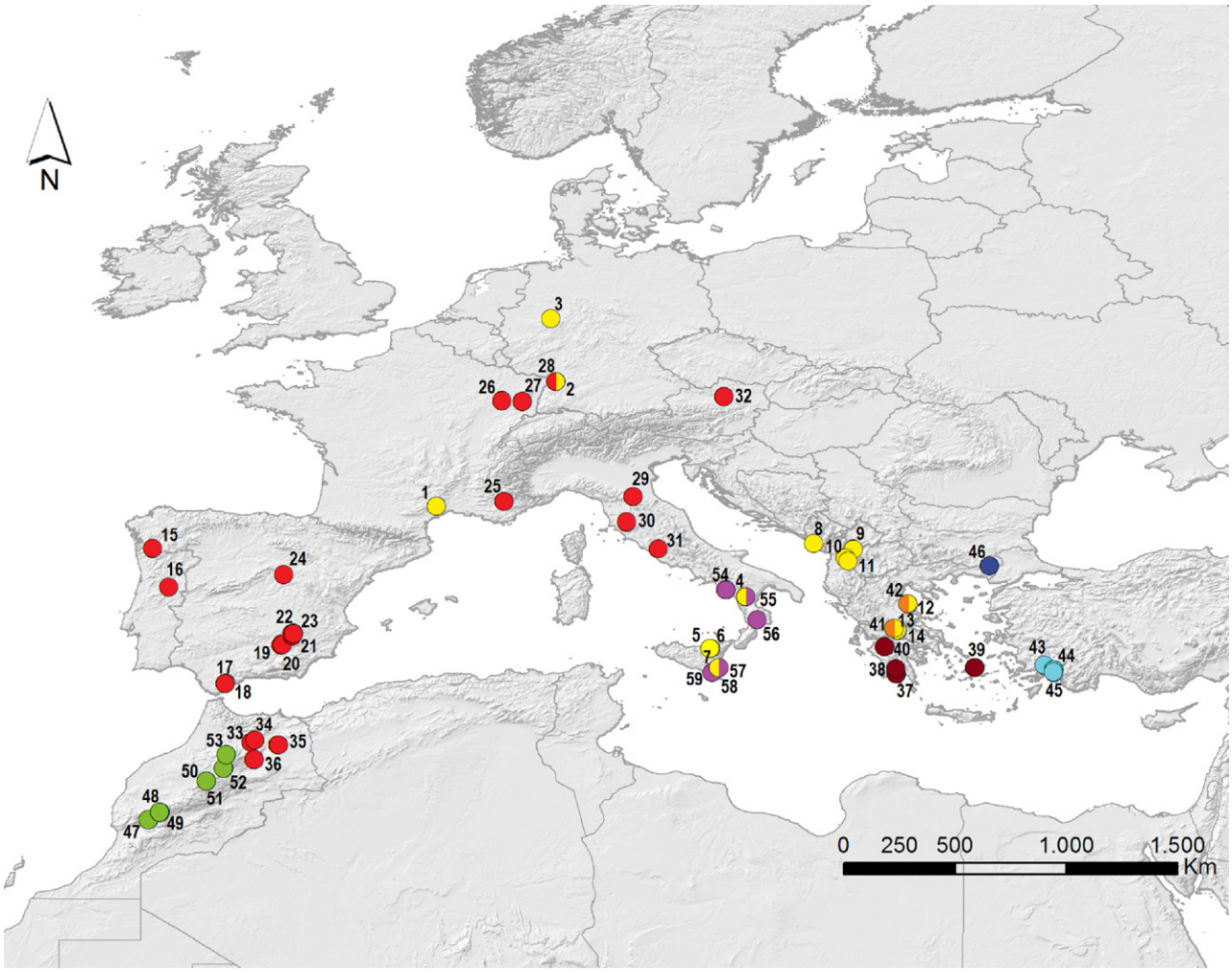

Figure 1. Map showing the location of sequenced samples of *Cordulegaster* species in the Western Palaearctic. Population codes match those used in Table 1. Yellow, *Cordulegaster bidentata*; red, *Cordulegaster boltonii*; green, *Cordulegaster princeps*; violet, *Cordulegaster trinacriae*; orange, *Cordulegaster heros*; brown, *Cordulegaster helladica*; light blue, *Cordulegaster insignis*; dark blue, *Cordulegaster picta*.

parameter estimation values using TRACER, version 1.5 (Rambaut & Drummond, 2003).

Estimates of average evolutionary divergence over sequence pairs within and between groups, using uncorrected *p*-distances (*p*-dist), were calculated based on the number of base differences per site from averaging over all sequence pairs within each group and estimation of net average between groups of sequences, respectively in MEGA, version 4.0.2 (Tamura *et al*., 2007).

Sequences from ITS-1 were aligned in MAFFT (Katoh & Miyata, 2005) and the alignment was analyzed using ML and BI methods, in accordance with the same methodology for the COI fragment. Because these sequences were found to be generally conserved (preliminary results; data not shown), unrooted networks were constructed using statistical parsimony (Templeton, Crandall & Sing, 1992) to evaluate the relationships among these closely-related haplotypes. This analysis was implemented in TCS, version 1.21 (Clement, Posada & Crandall, 2000) with a connection limit of 95% and indels treated as a fifth state. The same methodology was used with COI in order to better evaluate variation within species.

RESULTS

The COI alignment consisted of 112 DNA sequences from *Cordulegaster* specimens and six outgroup specimens (Table 1). Aligned sequences had a total length of 570 bp, with 163 polymorphic and 139 parsimony informative sites and high levels of nucleotide variability (H_D = 0.965, π = 0.06349, within *Cordulegaster*). No

Table 1. List of samples: species, subspecies, number of specimens (*N*), population code, country, IUCN regional Red List threatened categories (European/North African), and cytochrome *c* oxidase I (COI) and Internal Transcribed Spacer-1 (ITS-1) haplotypes

						Haplotype	
Species	Subspecies	*N*	Population	Country	IUCN	COI	ITS
Cordulegaster bidentata	*bidentata*	5	1, 4	France, Italy	NT	h1, h2, h3, h4	Q
Cordulegaster bidentata	*sicilica*	4	5–7	Italy Sicily	NT	h5, h6, h7	O
Cordulegaster bidentata		13	2–3, 8–14	Germany, Montenegro, Albania, Greece	NT	h2, h8–h15	O, P
Cordulegaster boltonii	*iberica*	10	18–23	Spain	LC	h19–h24	E, F
Cordulegaster boltonii	*immaculifrons*	5	25	France, Portugal	LC	h16, h21, h25	A, B
Cordulegaster boltonii	*boltonii*	4	16	France, Portugal	LC	h17, h21, h26	A, B, E
Cordulegaster boltonii		11	24, 28–32	Austria, Germany, Italy, Spain	LC	h21, h27–h28	C, D
Cordulegaster boltonii	*algirica*	12	17, 33–36	Morocco, Spain	LC	h18, h29–h31	E, G
Cordulegaster helladica	*helladica*	3	37, 38	Greece	EN	h32–h33	S, T
Cordulegaster helladica	*buchholzi*	6	39	Greece	EN	h34	R
Cordulegaster helladica		3	40	Greece	EN	h35	L, S
Cordulegaster heros	*pelionensis*	3	41–42	Greece	NT	h37	L
Cordulegaster insignis		6	43–45	Turkey	EN	h38–h40	R
Cordulegaster picta		2	46	Greece	VU	h41	M, N
Cordulegaster princeps		17	47–53	Morocco	NT	h42–h49	H, I, J
Cordulegaster trinacriae		8	54–59	Italy	NT	h50–h51	K
Anotogaster sieboldi		1		Japan			
Chlorogomphus brunneus		1		Japan			
Chlorogomphus iriomotensis		1		Japan			
Chlorogomphus okinawensis		1		Japan			
Anax imperator		1		Portugal			
Onychogomphus forcipatus		1		Morocco			

indels and no stop codons were observed, after translating all sequences to amino acids.

The final COI alignment used in the phylogeny reconstruction consisted of 57 distinct haplotypes, including the outgroups. The tree topologies resulting from ML and BI approaches were congruent and received high support values (> 0.9) for most relationships between species (Fig. 2). The *Cordulegaster* genus is clearly separated into two distinct clades, corresponding to the *boltonii*- and *bidentata*-groups and monophyly is evident with strong nodal support for all the species, with the relationships between species in the *boltonii*-group being better resolved (Fig. 2). The *boltonii*-group includes five *Cordulegaster* species: *Cordulegaster boltonii*, *Cordulegaster trinacriae*, *Cordulegaster princeps*, *Cordulegaster heros* and *Cordulegaster picta*, whereas *Cordulegaster bidentata*, *Cordulegaster insignis* and *Cordulegaster helladica* grouped together to form the *bidentata*-group. Relationships within the *boltonii*-group are better resolved in the BI analysis than in the ML; furthermore, *C. boltonii* and *C. trinacriae* are sister taxa (89 Bayesian posterior probability but no bootstrap support), with the Moroccan endemic *C. princeps* being related to this pair (again with higher Bayesian posterior probability than bootstrap support). *Cordulegaster heros* and *C. picta*, from the Balkans and Turkey are sister taxa, basally related to the three previous members of the group. Within the *bidentata*-group, relationships between the three species were not well supported in both BI and ML analysis. The maximum pairwise sequence divergence (uncorrected distances) was retrieved between *C. princeps* and *C. bidentata* (10%) and the minimum value (5.1%) was observed between *C. helladica* and *C. insignis* (Table 2).

Although the number of haplotypes found within the more widespread species *C. boltonii* and *C. bidentata* was quite high (16 and 15, respectively), the haplotype networks showed little correspondence with either geography or currently defined subspecies. Within *C. boltonii*, a notable exception are the specimens of *Cordulegaster boltonii algirica* from North Africa, which form a distinct group separated from all other haplotypes (h29, h30 and h31) (Fig. 3). Specimens from *Cordulegaster boltonii iberica* and *Cordulegaster boltonii immaculifrons* did not form distinct units and shared the commonest haplotypes. The network has the classical star topology with a central haplotype (h21) shared by the three other subspecies: *Cordulegaster boltonii boltonii*, *C. b. iberica* and *C. b. immaculifrons* (Fig. 3). Moreover, h21 is present in individuals from several locations in Iberia, France, Germany and Austria covering most of the range of the species. The four specimens from central Italy (Tuscany) are represented by two exclusive haplotypes (h27 and h28)

Table 2. Estimates of evolutionary divergence over sequence pairs between groups (A) (number of base differences per site from averaging over all sequence pairs between groups (SE estimates are shown above the diagonal) and estimates of average evolutionary divergence over sequence pairs within groups (B) (number of base differences per site from averaging over all sequence pairs within each group)

A	*Cordulegaster boltonii*	*Cordulegaster trinacriae*	*Cordulegaster princeps*	*Cordulegaster heros*	*Cordulegaster picta*	*Cordulegaster bidentata*	*Cordulegaster insignis*	*Cordulegaster helladica*	B
Cordulegaster boltonii	–	0.009	0.009	0.011	0.01	0.011	0.011	0.011	0.021
Cordulegaster trinacriae	0.053	–	0.009	0.011	0.01	0.011	0.011	0.011	0.002
Cordulegaster princeps	0.055	0.057	–	0.011	0.01	0.011	0.011	0.01	0.009
Cordulegaster heros	0.079	0.082	0.08	–	0.009	0.011	0.01	0.01	0
Cordulegaster picta	0.073	0.071	0.08	0.054	–	0.011	0.01	0.01	0
Cordulegaster bidentata	0.091	0.081	0.1	0.09	0.092	–	0.009	0.009	0.019
Cordulegaster insignis	0.092	0.075	0.093	0.081	0.081	0.062	–	0.008	0.011
Cordulegaster helladica	0.087	0.081	0.089	0.081	0.079	0.062	0.051	–	0.04

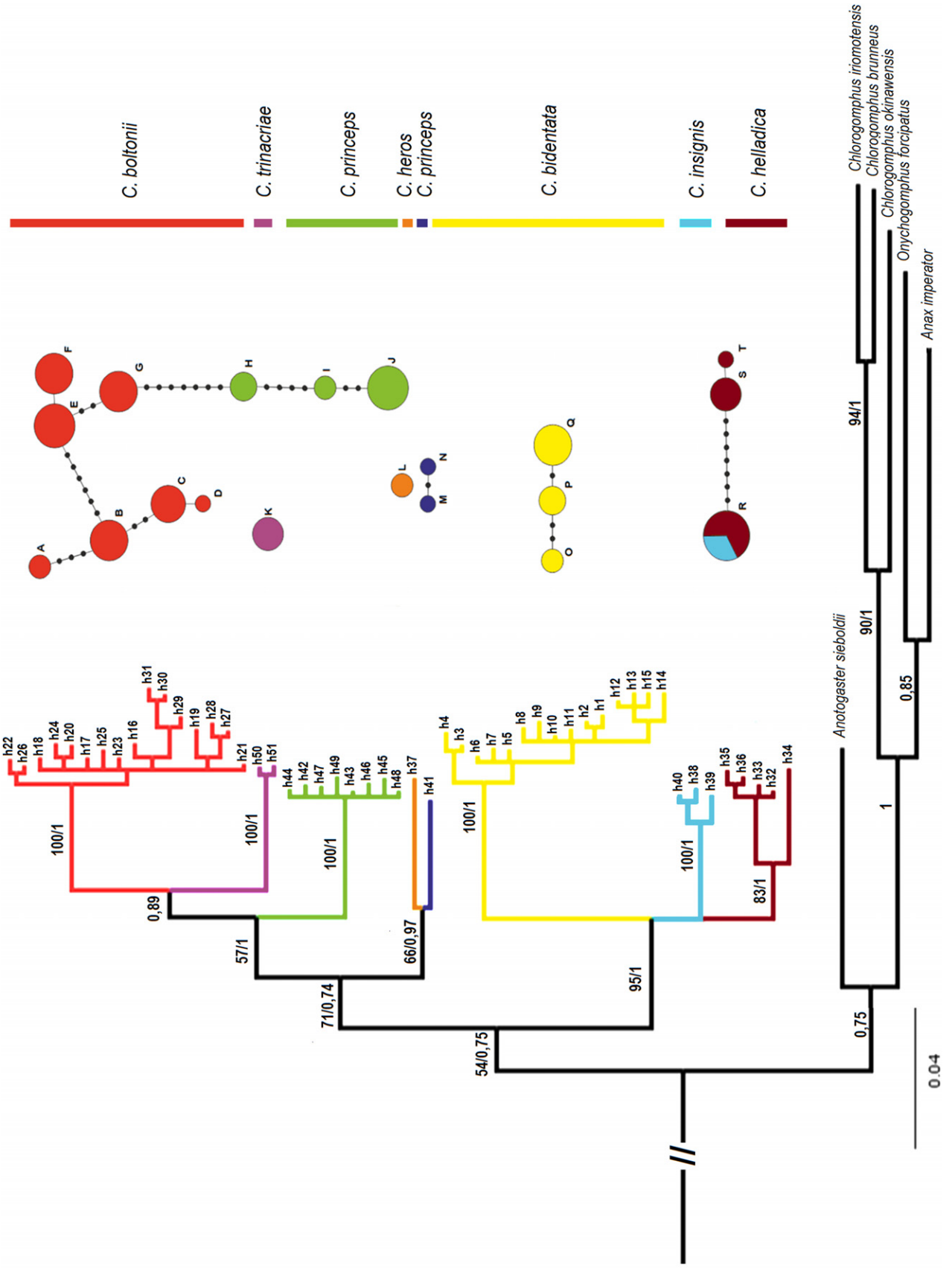
C. boltonii
C. trinacriae
C. princeps
C. heros
C. princeps
C. bidentata
C. insignis
C. helladica
Chlorogomphus iriomotensis
Chlorogomphus brunneus
Chlorogomphus okinawensis
Onychogomphus forcipatus
Anax imperator
Anotogaster sieboldii
94/1
90/1
1
0,85
0,75
54/0,75
71/0,74
57/1
0,89
66/0,97
95/1
100/1
100/1
100/1
100/1
100/1
83/1
0.04

Figure 2. Left: phylogenetic tree obtained by Bayesian inference (BI) analysis of 112 cytochrome *c* oxidase I sequences (570 bp) of *Cordulegaster* specimens and several outgroups. Support values (%) are given as bootstrap support/Bayesian posterior probability. The tree topologies resulting from Maximum Likelihood and BI approaches were congruent. Right: haplotype (TCS) networks showing the relationships of *Cordulegaster* specimens, inferred from 79 individuals sequenced for 490-bp ITS nuclear sequences. Circle size is proportional to the observed haplotype frequencies and black points represent unobserved haplotypes and potential intermediates.

exhibiting some distinctiveness (three steps). The *C. bidentata* haplotype network does not corroborate the distinctiveness of *Cordulegaster bidenta sicilica* (h5, h6 and h7) from the nominotypical subspecies and indicates the specimens from South Italy (h3 and h4) as the most genetically distinct ones (five mutations) (Fig. 3). The haplotypes of *Cordulegaster helladica helladica* and *Cordulegaster helladica buchholzi* do not connect and are not shared with *C. insignis*.

The ITS-1 alignment consisted of 79 DNA sequences from *Cordulegaster* specimens (Table 1), as a result of the non-amplification of some individuals. Aligned sequences had a total length of 490 bp with no heterozygote individuals found; regarding indels, two 4–8-bp indels were found within the *boltonii*-group and five 2–8-bp indels within the *bidentata*-group. The diversity observed in the ITS-1 sequences in general supports the species distinctions estimated with the COI sequence data, with one noteworthy exception: all the *C. h. buchholzi* analyzed share the haplotype with *C. insignis* (haplotype R) (Fig. 2). Furthermore, only two pairs of species are connected under the 95% parsimony criterion used, by a minimum of eight steps: *C. boltonii* and *C. princeps*, and *C. insignis* and *C. helladica* (Fig. 2).

DISCUSSION

The European Odonata fauna is particularly well known, yet taxonomic uncertainties remain in several genera (Dijkstra & Kalkman, 2012), including *Cordulegaster*. We examined for the first time both mitochondrial and nuclear genes sequences for all Western Palaearctic species of this genus. Our data support the existence of two major groups corresponding to the *boltonii*- and *bidentata*-groups in agreement with morphological and ecological traits. Furthermore, all species are monophyletic and the diversity observed in the ITS-1 sequences supports the species distinctions estimated with the COI sequence data, with one notable exception: all the *C. h. buchholzi* analyzed share the haplotype with *C. insignis* from Turkey. *Cordulegaster helladica* and *C. insignis* are morphologically distinct species, being identified by the shape of the posterior face of the occipital triangle (Lohmann, 1993). They occur in relatively close geographical areas (populations 39, 43–45; Fig. 1) and therefore this shared haplotype might be the result of hybridization, or the maintenance of an ancestral polymorphism. However, the low number of the specimens analyzed does not allow a more comprehensive assessment of the taxonomic implications of this shared haplotype.

Regarding subspecies validity and within the widespread *C. boltonii*, only the North African populations of *C. b. algirica* are genetically distinct, with the European specimen not grouping together, despite its morphological similarity. Specimens from *C. b. iberica* and *C. b. immaculifrons* did not form distinct units and shared the commonest haplotypes. The value of maintaining these taxa at the subspecies level is doubtful. Although various studies address the usefulness of subspecies (Braby, Eastwood & Murray, 2012), in the case of these forms that show genetic overlap and only colour pattern variations, there is little obvious taxonomic or conservation value. The validity of *C. bidenta sicilica* is also questionable because our data do not corroborate its distinctiveness from *C. bidentata bidentata.* By contrast, there are two haplogroups separated by 4.0% divergence (COI) in *C. helladica*, namely *C. h. helladica* from the Peloponnese and *C. h. buchholzi* from the Cyclades islands. This divergence is the highest observed within a species in the present study, and at a level similar to that between some accepted species. This result, together with the IUCN Red List of threatened categories of *C. helladica* and its subspecies status (Boudot, 2010; Kalkman *et al.*, 2010), reinforces the need of more sampling and the use of additional markers for a detailed assessment of the Greek and Turkish *Cordulegaster* taxa. This would allow clarifying conservation priorities and assisting the development of adequate conservation measures.

It appears likely that North Africa was colonized twice from Iberia, with one older colonization by a common ancestor of *C. princeps* and *C. boltonii* and one more recent by *C. boltonii*. Other taxa, such as wall lizards (*Podarcis* spp.) show a similar pattern (Kaliontzopoulou *et al.*, 2011), although the wide variety of colonization patterns across the Straits for different organisms (e.g. Habel, Dieker & Schmitt, 2009; Habel *et al.*, 2010) have led to the hypothesis that evolutionary histories unique to each species have produced this array of diverse scenarios (Jaramillo-Correa *et al.*, 2010). As a result of the wide distribution of the commonest haplotypes, especially

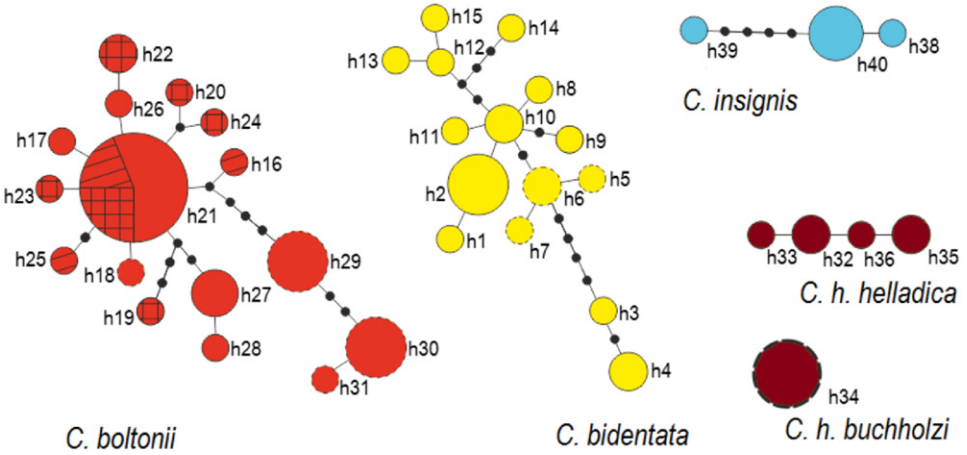

Figure 3. Haplotype (TCS) networks showing the relationships of *Cordulegaster* specimens, inferred from 82 individuals sequenced for 570-bp cytochrome *c* oxidase I (COI) sequences, and the subspecies distinctiveness. Circle size is proportional to the observed haplotype frequencies and black points represent unobserved haplotypes and potential intermediates. Red, parsimony network of the 16 haplotypes observed in *Cordulegaster boltonii*: plain, *Cordulegaster boltonii boltonii*; dashed line, *Cordulegaster boltonii algirica*; cross hatched, *Cordulegaster immaculifrons*; squares, *Cordulegaster boltonii iberica.* Yellow: parsimony network of the 15 haplotypes observed in *Cordulegaster bidentata*: plain, *Cordulegaster bidentata bidentata*; dashed, *Cordulegaster bidentata sicilica*. Light blue: parsimony network of the three haplotypes observed in *Cordulegaster insignis.* Brown: parsimony networks of COI 5 haplotypes observed in *Cordulegaster helladica*: plain, *Cordulegaster helladica helladica*; dashed, *Cordulegaster helladica buchholzi*. For individual codes, see Table 1.

for *C. boltonii*, determination of refugium locations is difficult without further sampling and the use of faster evolving markers.

To conclude, our results generally support the current taxonomy of *Cordulegaster* in the Western Palaearctic, although particularly in Greece a detailed study is needed to determine the taxonomic status of the endangered subspecies, which show a high level of differentiation in COI but also an incongruent pattern with ITS-1. The two ecological groups, the *boltonii-* and the *bidentata*-groups, are well-supported genetic entities, with relationships between species in the *boltonii*-group being better resolved. Moderate genetic diversity within *C. bidentata* and *C. boltonii* species suggests that large populations survived in diverse refugia during the last ice ages, although little geographical differentiation/substructuration is presently observed. No evidence was found supporting the validity of *C. b. iberica*, *C. b. immaculifrons* and *C. bidenta sicilica*, thus, conservation efforts can be appropriately focused at the species level in Europe for both *C. boltonii and C. bidentata*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of samples: species, sample collection code, collection sites (population, latitude, longitude and country), and GenBank accession codes.